Tumor and Stem Cell Biology

Dishevelled 2 Signaling Promotes Self-Renewal and Tumorigenicity in Human Gliomas

Teodoro Pulvirenti1, Maartje Van Der Heijden2, Leif A. Droms2, Jason T. Huse3, Viviane Tabar2, and Alan Hall1

Abstract

Glioblastoma multiforme is the most common glioma variant in adults and is highly malignant. Tumors are thought to harbor a subpopulation of stem-like cancer cells, with the bulk resembling neural progenitor-like cells that are unable to fully differentiate. Although multiple pathways are known to be involved in glioma tumorigenesis, the role of Wnt signaling has been poorly described. Here, we show that Dishevelled 2 (Dvl2), a key component of the Wnt signaling pathway, is overexpressed in human gliomas. RNA interference–mediated depletion of Dvl2 blocked proliferation and promoted the differentiation of cultured human glioma cell lines and primary, patient-derived glioma cells. In addition, Dvl2 depletion inhibited tumor formation after intracranial injection of glioblastoma cells in immunodeficient mice. Inhibition of canonical Wnt/β-catenin signaling also blocked proliferation, but unlike Dvl2 depletion, did not induce differentiation. Finally, Wnt5a, a noncanonical Wnt ligand, was also required for glioma cell proliferation. The data therefore suggest that both canonical and noncanonical Wnt signaling pathways downstream of Dvl2 cooperate to maintain the proliferative capacity of human glioblastomas. Cancer Res; 71(23); 7280–90. ©2011 AACR.

Introduction

Glioblastoma multiforme (GBM) is a lethal brain tumor, with most patients dying within 1 year of diagnosis (1, 2). The last decade has witnessed very little advance in treatment, but the identification of stem-like cancer cells in brain tumors has provided new insights into the disease. Several groups have identified a subpopulation of stem-like cancer cells in brain tumors, using biomarker analysis and culturing techniques similar to those used to characterize normal neural stem cells. These cells show a significant tumor-initiating ability, are capable of self-renewal, and express neural stem cell markers, such as Nestin and CD133, but not markers of the differentiated neural lineage (3–7). Although these cells represent only a small fraction of the tumor bulk, their high self-renewal capacity is thought to sustain tumor growth. The identification of signaling pathways that maintain the proliferative capacity of these cells and/or regulate their decision to differentiate offers great potential for a better understanding of the disease at the molecular level.

For neural stem cells, the decision to divide or differentiate is strongly influenced by Wnt signaling, whereas other factors, such as epidermal growth factor (EGF) and fibroblast growth factor (FGF), stimulate their proliferation (8–10). Wnt ligands are capable of activating several distinct signal transduction pathways and promoting a large spectrum of cellular processes such as proliferation, differentiation, polarity, adhesion and migration (11, 12). Wnt signaling pathways are usually categorized as canonical or noncanonical. The former describes the classical β-catenin/gene transcription pathway, whereas the latter is most often linked with polarity establishment and cytoskeleton-mediated processes but can also involve gene transcriptional effects (though not through β-catenin). The scaffold protein Dishevelled (Dvl) plays an essential role in all known Wnt signaling pathways. Activation of canonical Wnt signaling recruits Dvl to the plasma membrane, resulting in the disassembly of the β-catenin destruction complex and leading to the translocation of active β-catenin to the nucleus, with subsequent activation of gene expression. Activation of noncanonical Wnt pathways leads to Dvl-mediated activation of Rho GTPases, planar cell polarity proteins, and Ca2+-dependent signals (13–17). To discriminate between canonical and noncanonical Wnt pathways, Wnt signaling uses different domains of Dvl to activate distinct downstream components. In particular, the DIX domain is responsible for binding to Axin and activation of the canonical/β-catenin pathway, whereas the DEP domain functions only in the noncanonical Wnt pathway and is responsible for activation of the small GTPases, Rho and Rac (18).

Mutations in the genes encoding components of Wnt signaling pathways have been found in many human cancers, notably colorectal cancer (13, 19). Mutations in Axin, β-catenin, and adenomatous polyposis coli (APC) are found in sporadic medulloblastomas along with nuclear localization of β-catenin, suggesting constitutive Wnt signaling (20–22). Wnt signaling through the canonical β-catenin pathway has also been
reported to increase the stem-like behavior of astrocytes and glioma cell lines, whereas downregulation of canonical Wnt/β-catenin pathway induces apoptosis in glioma cell lines (23, 24). High expression levels of Dvl2 have so far been observed in both lung and colon cancers. Dvl overexpression has been shown to be critical for Wnt signaling in non–small cell lung cancer (25, 26). Dvl has also been implicated, together with mTOR, in the progression of colorectal neoplasia (27).

In this study, we examine the role of Dvl in human gliomas. A tissue microarray (TMA) analysis revealed Dvl2 overexpression in more than 70% of the analyzed GBM samples. Dvl2 depletion was found to block the proliferation of human gliomas and promote their differentiation both in vitro and in vivo. Finally, both canonical and noncanonical signaling pathways are required to maintain the proliferative capacity of glioma cells.

Materials and Methods

**Animal experiments**

Cells were infected with viral vectors and selected in puromycin as described later. Six- to 8-week-old nude mice were injected intracranially with 10^6 cells into the striatum under aseptic conditions. Mice were maintained until the occurrence of neurologic signs and then sacrificed and perfused fixed with 4% paraformaldehyde (PFA). The brains were postfixed for 24 hours in 4% PFA, incubated in 30% sucrose, and then snap frozen in optimal cutting temperature (OCT) compound and cut on a cryostat (coronal sections, 25 μm). Standard protocols were used for hematoxylin and eosin staining. Immunofluorescence staining was conducted as described in the Immunofluorescence analysis section.

**TMA and tumor samples**

For analysis of Dvl2 levels by immunohistochemistry, a tissue TMA containing glioblastoma and normal brain samples was used (US Biomax, GL806). The TMA contains 35 cases of glioblastoma and 5 normal brain tissues, represented as duplicate cores per case. The immunohistochemical detection of Dvl2 was conducted at the Molecular Cytology Core Facility of MSKCC using Discovery XT processor (Ventana Medical Systems). Tissue sections were blocked for 30 minutes in 10% normal goat serum in 0.2% bovine serum albumin/PBS, followed by incubation for 5 hours with 10 μg/mL of the primary antibody (rabbit polyclonal anti-Dvl-2; Chemicon cat# ab3972 lot# j61682917) and incubation for 60 minutes with biotinylated goat anti-rabbit IgG (Vector Labs, cat#: PK6101; 1:200 dilution). The detection was conducted with DAB-MAP kit (Ventana Medical Systems). The glioblastoma samples for Western blot analysis were collected from patients undergoing surgery at MSKCC, with consent. Normal tissue was obtained from a small cortical area removed during the surgical approach. The samples were snap frozen in liquid nitrogen, triturated using a plastic pestle, and then collected in lysis buffer as described (see Protein analysis in Supplementary Material).

**Cell lines and human GBMs**

The human glioma cell lines U87, U138, U373, and U251 were cultured in MEM Gluta-Max (Gibco, 41090) supplemented with 10% fetal calf serum (FCS). 10 mmol/L HEPEs, nonessential amino acids (Gibco, 11140), and antibiotics. LN229 were cultured in Dulbecco’s Modified Eagle’s Media supplemented with 5% FCS and antibiotics. Primary glioblastoma samples were derived from patients undergoing surgery at MSKCC. Primary GBM samples were dissociated as described by Pollard and colleagues and grown as a monolayer on plastic culture dishes coated with 10 ng/mL laminin (Sigma, L2020; ref. 28). All patient-derived cells were cultured in NeuroCult NS-A basal medium, human (StemCell Technology, 05750), supplemented with NeuroCult NS-A proliferation supplements, human (StemCell Technology, 05754), and 10 ng/mL human basic FGF (Sigma, F0291) and 20 ng/mL human EGF (Prepotech, AF-100-15; In the main text, we refer to this medium as neural stem cell medium for simplicity.) For differentiation, primary GBM cells were plated on 10 ng/mL laminin, and the NeuroCult NS-A basal medium was replaced with MEM Gluta-Max 5% FCS 48 hours before the infection.

**Growth curve and neurosphere formation assay**

Cells were infected with control or Dvl2 short hairpin RNA (shRNA) and selected in 1 μg/mL puromycin for 5 days. A total of 2 × 10^4 cells were plated per well in a 12-well plate, and each sample was plated in triplicate. For glioma cell lines growing in 10% FCS, cell number was counted after 3, 5, and 7 days. Primary GBM cells were plated on laminin-coated dishes and grown as an adherent monolayer in serum-free neural stem cell medium. Cells were counted after 4, 8, and 16 days. For neurosphere formation, cells were grown and infected in an adherent monolayer in neural stem cell medium. After puromycin selection, cells were resuspended and grown in neural stem cell medium containing 0.7% methylcellulose (9). A total of 5 × 10^3 cells were plated in each well of a 24-well plate in quadruplicate. The number of neurospheres with a diameter greater than 50 μm was counted after 10 or 15 days, for U87 or primary GBMs, respectively.

**Immunofluorescence analysis**

Cells were plated on glass coverslips coated with laminin and infected with viral vectors as described above. After selection, cells were fixed in 4% formaldehyde, rinsed 3 times in 1× PBS, and permeabilized in PBS containing 0.3% Triton and 10% goat serum. Samples were incubated overnight at 4°C with the primary antibody and 45 minutes at room temperature with the secondary antibody and with Hoechst. Mouse brain cryosections were stained following the same procedure. For fluorescence imaging, images were taken using 10× or 20× objective lenses on a Zeiss Axio Imager.1A microscope. The following antibodies were used: mouse anti-Nestin (Abcam, ab22035; 1:100), mouse anti-Tuj1 (Millipore, MAB1637; 1:250), rabbit anti-GFAP (DAKO, Z0334; 1:100), mouse anti-Tuj1 (Millipore, MAB1637; 1:250), rabbit anti-GFAP (DAKO, Z0334; 1:100), mouse anti-Tuj1 (Millipore, MAB1637; 1:250), and rabbit anti-GFAP (DAKO, Z0334; 1:100). For actin cytoskeleton detection, Alexa 546–conjugated phallidin was used (Invitrogen, A22833; 1:100). Apoptosis was detected on fixed cells using the In Situ Cell Death Detection Kit (Roche, 11684795910), according to the manufacturer’s instructions.
Senescence was detected using the Senescence β-galactosidase Kit (Cell Signaling, 9860S), following the manufacturer's instructions.

Statistical analysis
Statistical analysis was conducted using Microsoft Excel 2008. The Student t test (2-tailed, unpaired) was used to determine the significance of results comparing cells infected with shControl and Dvl2 shRNA. Wilcoxon–Mann–Whitney test was used to determine the significance of the in vivo tumor growth.

Results
Endogenous Dishevelled is expressed at high levels in human glioblastomas
Overexpression of Dvl has been shown to potentiate the activation of Wnt signaling pathways (29, 30). To examine the potential role of Wnt signaling in high-grade brain tumors, we first compared the expression levels of Dvl2 (the most widely expressed isoform of Dishevelled) in normal and cancer brain tissues using the Oncomine database. Analysis based on a set of data including 80 glioblastoma samples showed that the levels of Dvl2 mRNA were increased in brain cancer tissue compared with normal tissue (Fig. 1A; ref. 31). The Cancer Genome Atlas (TCGA) data set was also analyzed for Dvl2 expression in brain cancer but the results appeared nonsignificant (P = 1.000; not shown). The level of Dvl2 protein was next analyzed in a group of 10 freshly-derived GBM samples. As shown in Fig. 1B, Dvl2 is overexpressed, though to different extents, in all patient samples, when compared with normal brain tissue. EGFR overexpression and p53 loss are commonly found in human GBMs, but no significant correlation was found between Dvl2 and either EGFR or p53 expression (Fig. 1B; refs. 2, 32, 33). We also analyzed the status of isocitrate dehydrogenase-1 (IDH1) gene in these freshly derived samples (34). As shown in Supplementary Fig. S1, only one sample showed a mutation in IDH1, the mutation being the less common R132G.

To further investigate the expression levels of Dvl2 in high-grade gliomas, a TMA containing 35 samples from patients diagnosed with grade IV glioma and 5 control brain samples was examined using a Dvl2-specific antibody. Tumors were scored as negative (0), medium positive (1), or very positive (2). Dvl2 is overexpressed in more than 70% of the GBM samples, with around 20% of these showing very high levels (Fig. 1C). We conclude that Dvl2 is overexpressed in a significant number of human GBM samples, raising the possibility that Wnt signaling plays an important role in these tumors.

Figure 1. Dvl2 is overexpressed in human glioblastomas. A, Oncomine microarray data analysis for Dvl2 expression in glioblastoma versus normal brain tissue is shown. The Student t test was conducted using the Oncomine software; P = 0.001. The boxes represent the 25th through 75th percentiles; the horizontal lines represent the medians; the points represent the end of the ranges. B, expression of Dvl2 in normal brain and 11 fresh-derived GBM samples, analyzed by Western blot. The levels of expression of EGFR and p53 were also analyzed. Normal tissue was obtained from a small cortical area removed during the surgical approach. C, immunohistochemistry of Dvl2 on TMA containing 35 GBM samples and 5 normal brain samples. Scores (0), (1), and (2) represent negative, medium positive, and highly positive staining, respectively. Top, representative staining of normal brain and 2 GBM cases; scale bar, 100 μm; bottom, a magnification of the top; scale bar, 20 μm.
Dishevelled depletion blocks proliferation and induces differentiation of U87 glioma cells

To explore the role of Wnt signaling in human GBM, lentiviral shRNA vectors targeting Dvl2, which is essential for all known Wnt signaling pathways, were obtained (14). The glioma cell line U87, originally derived from a human glioblastoma, harbors mutations in PTEN and p16^ink4a and is highly proliferative and tumorigenic both in vivo and in vitro. Two different lentiviral constructs, which efficiently deplete Dvl2 in these cells significantly inhibited their proliferation...
Dishevelled depletion induces the differentiation of primary, patient-derived glioblastoma cultures

To determine whether Dvl2 plays an important role in primary human glioblastoma, freshly derived tumor samples were obtained from 3 individual patients (GBM1/GBM2/GBM3; Supplementary Table S1 and Fig. S5). Tumors were dissociated and grown as adherent cultures on laminin-coated dishes (28). GBM cells were then infected with lentiviral vectors targeting Dvl2 or a control shRNA. Dvl2 depletion inhibited the proliferation of all 3 GBMs in serum-free cultures (Fig. 4), as well as their ability to grow as neurospheres in neural stem cell medium (Fig. 5H). All 3 GBMs underwent significant morphologic changes reminiscent of differentiation, together with downregulation of Nestin and upregulation of the glial marker GFAP (Fig. 5A–G). A significant increase in p21<sup>WAF</sup> and Tuj1 was also seen by Western blot analysis in 2 of 3 samples (Fig. 5G). We conclude that Dvl2-mediated signaling is required to maintain the self-renewal ability of both glioma cell lines and patient-derived GBM samples.
Dishevelled depletion inhibits glioblastoma tumorigenicity in vivo

To determine whether Dvl2 depletion is effective at suppressing in vivo tumorigenicity, intracranial injections of controls, or tumor cells originating from a patient tumor (GBM1), or cell lines (including a GFP-U87 line) expressing Dvl2 shRNA were conducted in NOD/SCID mice (for a total of 8 control mice and 12 mice injected with Dvl2-depleted cells). MRI conducted 13 weeks after injection of cells derived from patient GBM1 revealed an extensive and invasive tumor mass (Fig. 6A). However, GBM1 cells that had been infected with Dvl2 shRNA lentivirus 7 days prior to intracranial injection did not produce tumors detectable by MRI or histology at this time (Fig. 6A). To determine whether Dvl2 depletion is effective at suppressing in vivo tumorigenicity, intracranial injections of controls, or tumor cells originating from a patient tumor (GBM1), or cell lines (including a GFP-U87 line) expressing Dvl2 shRNA were conducted in NOD/SCID mice (for a total of 8 control mice and 12 mice injected with Dvl2-depleted cells). MRI conducted 13 weeks after injection of cells derived from patient GBM1 revealed an extensive and invasive tumor mass (Fig. 6A). However, GBM1 cells that had been infected with Dvl2 shRNA lentivirus 7 days prior to intracranial injection did not produce tumors detectable by MRI or histology at this time (Fig. 6A). In 1 of 3 mice injected with Dvl2-depleted GBM1, a small lesion was detectable by MRI or histology at this time (Fig. 6A). In 1 of 3 mice injected with Dvl2-depleted GBM1, a small lesion was detectable by MRI or histology at this time (Fig. 6A). In 1 of 3 mice injected with Dvl2-depleted GBM1, a small lesion was detectable by MRI or histology at this time (Fig. 6A). In 1 of 3 mice injected with Dvl2-depleted GBM1, a small lesion was detectable by MRI or histology at this time (Fig. 6A).

To investigate the fate of Dvl2-depleted GBM1 cells in vivo, brain cryosections were obtained. Immunohistologic studies showed a highly proliferative tumor mass in mice injected with control but not Dvl2-depleted cells (Fig. 6B). Similarly, using an antibody specific for human Nestin, rare human GBM1 cells were found at the Dvl2-depleted injection sites (Fig. 6C). The small lesion found in 1 of 12 mice injected with Dvl2-depleted cells was positive for human Nestin (not shown).

To determine whether Dvl2-depleted cells die after injection, or adopt a differentiated phenotype and integrate into the mouse brain, a GFP-expressing U87 cell line (U87-GFP) was generated. Control cells (U87-GFP) generated a tumor 5 weeks after intracranial injection, whereas Dvl2-depleted U87-GFP cells induced no lesions detectable by MRI up to 20 weeks after injection (Fig. 6C; Supplementary Fig. S8A and S8B). Subsequent analysis of brain cryosections for GFAP (an astrocytic differentiation marker) and GFP revealed cells that had infiltrated the mouse brain and were positive for both (Fig. 6D and E, arrows). This result indicates that the injected Dvl2-depleted cells are still present in the mouse brain and some adopt a differentiation-like state (Fig. 6D and E).

Canonical and noncanonical Wnt signaling pathways cooperate in the regulation of glioblastoma cell proliferation

Although different Wnt ligands can activate distinct signal transduction pathways, Dishevelled is thought to be essential in all cases (14). To begin to explore the nature of Dvl2 signaling in glioblastoma, we used U87 cells. siRNA oligonucleotides were used to deplete β-catenin, an essential component of the canonical Wnt signaling pathway. Despite a relatively modest RNAi knockdown (Fig. 7B), β-catenin depletion strongly inhibited the proliferation of U87 cells in culture (Fig. 7A). However, it did not induce differentiation as judged by morphology or protein markers (p21WNT1/Nestin/Tuj1; Fig. 7B: Supplementary Fig. S9B). As an alternative approach to inhibit canonical Wnt signaling, a truncated (dominant negative) form of the transcription factor TCF-4 (DN-TCF), previously shown to block β-catenin–mediated transcription, was used (37, 38). This also inhibited the proliferation of U87 cells (Fig. 7A) but did not induce any differentiation markers (Fig. 7B; Supplementary Fig. S9C). To further investigate the role of canonical Wnt/β-catenin signaling in the Dvl-dependent differentiation event, we used a non-phosphorylatable/active form of β-catenin. If the block of canonical Wnt/β-catenin signaling is responsible for the differentiation-like phenotype caused by Dvl2 depletion, an active form of β-catenin should be able to rescue this effect. As shown in Supplementary Fig. S10, U87 cells stably expressing active β-catenin (β-catenin with serine to alanine substitutions at positions 35, 37, 41, and 45, or β-catenin-S4A) were not able to rescue the proliferation block or the differentiation phenotype caused by Dvl2 depletion. We conclude that β-catenin–mediated, canonical Wnt signaling is required to maintain the proliferative capacity of human glioblastoma cells, as suggested by others (23). However, as loss of β-catenin/TCF-4 does not phenocopy the loss of Dvl2, and active β-catenin does not restore the normal proliferative behavior of these cells, we conclude that additional signals downstream of Dvl2 are also involved.

To investigate the possible involvement of noncanonical Wnt signaling, lentiviral constructs encoding shRNAs targeting the noncanonical Wnt ligand Wnt5a were used. Depletion of Wnt5a with 2 different shRNAs inhibited the proliferation of...
both U87 cells and primary cells derived from patient GBM1 (Fig. 7C–F) but was not able to induce differentiation (Fig. 7G). Furthermore, Wnt5a-depleted cells become flat and show high levels of β-galactosidase, a known marker of cellular senescence (Fig. 7H). Finally, depletion of Wnt5a dramatically inhibited the ability of primary GBM1 cells to form neurospheres in neural stem cell medium (Fig. 7F). These results suggest a role for a noncanonical Wnt pathway in regulating glioma cell proliferation.

Discussion

To explore the role of Wnt signaling in human glioblastoma, Dvl2, a key downstream component of all known Wnt signaling pathways, was depleted using RNAi. Dvl2 depletion not only inhibited the proliferation but also promoted the differentiation of human glioma cell lines as well as freshly derived patient glioblastoma samples. Dvl2 depletion inhibited neurosphere formation, an assay for the cancer stem-like subpopulation, as well as tumorigenicity, after intracranial injections in the mouse. Surprisingly, however, inhibition of the canonical Wnt signaling pathway, by β-catenin depletion, or expression of a dominant-negative version of the TCF transcription factor, blocked proliferation but did not induce differentiation. We have gone on to show that a noncanonical Wnt signaling pathway, involving the ligand Wnt5a, is also required to maintain the proliferative capacity of human glioblastoma cells. We conclude that a combination of canonical and
noncanonical Wnt signaling is required to maintain glioblastoma proliferation.

Human glioblastomas are associated with a variety of genetic changes, including mutations in PTEN, EGFR, PDGFR, p53, and p16(INK4a), and their contribution to disease development has been explored experimentally using mouse models (2, 39). Overexpression of platelet—derived growth factor receptor (PDGFR) in neural progenitors, for example, induces the formation of oligodendrogliomas (40), whereas inactivation of both p53 and PTEN prevents neural stem/progenitor cell differentiation, in part at least, through upregulation of c-myc and leads to the generation of malignant glioma (41). Other major signaling pathways involved in the regulation of neural stem cells have been reported to be altered in gliomas, including Notch and hedgehog (42). In fact, mutations in the Hedgehog signaling pathway have been found in another brain cancer, medulloblastoma, and inhibition of this pathway blocks the proliferation and self-renewal of stem-like glioma cells (43). Canonical Wnt signaling is a major player in maintaining the self-renewal capacity of neural stem cells and has been reported to be upregulated in brain cancers including glioma (10, 44, 45). A recent report identified PLAGL2 as a glioma oncogene and showed that it exerts its effects by promoting canonical Wnt

Figure 6. Dvl2 depletion suppresses tumorigenicity of primary GBM cells. GBM1 cells were infected with control and Dvl2 shRNA lentivirus as previously described and selected in puromycin for 5 days. A total of 2 x 10⁵ cells were stereotactically injected into the striatum of 3 NOD/SCID mice per group. A, tumor growth as determined by MRI 13 weeks after injection. Left, control cells; right, Dvl2-depleted cells. The asterisk in the left indicates the tumor. B, intracranial tumor characterization by hematoxylin and eosin (H&E) staining showing a high proliferative mass in mice injected with control cells than in Dvl2-depleted cells. C, immunofluorescence microscopy of brain cryosections. The cells were stained with an antibody specific for human Nestin to identify human cells in the mouse brain and Hoechst to visualize cell nuclei. Scale bar, 50 µm. D, U87 cells stably expressing GFP (U87-GFP) infected with control (left) and Dvl2 shRNA lentivirus (right) and injected into the striatum of NOD/SCID mice. A total of 2 x 10⁵ cells were injected per animal, and 5 mice were used for control and 9 for Dvl2-depletion. GFP was used to identify injected U87 cells. Cryosections were stained for the differentiation marker GFAP to determine cell fate in vivo. Scale bar, 100µm. E, enlargement of the area indicated by a dotted line in the right in (D) showing colocalization of GFP and GFAP markers (arrows). F, survival rate curve of NOD/SCID mice injected with 2 x 10⁵ control or Dvl2-depleted GBM1 cells (Wilcoxon–Mann–Whitney test, rank-sum test: P = 0.017). G, survival rate curve for mice injected with U87 cells infected with shControl of Dvl2shRNA lentivirus (Wilcoxon–Mann–Whitney rest, rank-sum test: P = 0.021). Data represent means ± SEM. Scale bar, 100 µm.
Figure 7. Canonical and noncanonical Wnt signaling maintain glioma cell proliferation. A, U87 cells were transfected with a nontargeting siRNA oligonucleotide (siControl) or an siRNA oligonucleotide targeting β-catenin, or infected with a control or a dominant-negative (DN)-TCF expressing retrovirus. Cells were plated in medium containing 10% FCS and cell numbers determined after 5 days. B, cells were treated as in (A), and Nestin, Tuj1 and p21WAF protein levels were determined by Western blot analysis. Dvl2-depleted U87 cells were used as a control. The number of neurospheres was expressed as percentage of control. C, U87 cells were infected with nontargeting shRNA lentivirus (shControl) or lentivirus expressing 2 distinct shRNAs targeting Wnt5a (Wnt5a sh1 and sh2). Cells were selected in puromycin for 5 days, plated in medium containing 10% FCS, and cell numbers determined after 5 days. D, Wnt5a mRNA depletion efficiency by the 2 shRNAs in U87 was determined by quantitative real-time PCR (qRT-PCR) and normalized to GAPDH expression. E, cells from patient GBM1 were infected with lentivirus containing nontargeting shRNA (shControl) or shRNAs directed against Wnt5a. Seven days after infection, cells were plated in neural stem cell medium as an adherent monolayer on laminin-coated dishes. Cell numbers were determined 8 days later. F, cells from patient GBM1 were treated as in (E) and the neurosphere formation ability determined 15 days after plating in neural stem cell medium containing 0.7% methylcellulose. The number of neurospheres was expressed as percentage of control. G, U87 cells were treated as in (C), and Nestin, Tuj1 and p21WAF protein levels were determined by Western blot analysis. Dvl2-depleted U87 cells were used as a control. H, cells were treated as in (C) and then fixed and stained for β-galactosidase (blue) to detect senescence. Data represent means ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001 relative to control cells (the Student t test). Scale bar, 100 μm. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
signaling to suppress differentiation and promote self-renewal of neural stem/progenitor cells (23).

Here, we show for the first time that Dvl2 is overexpressed in high-grade gliomas, suggesting a role for active Wnt signaling in regulating the biology of these tumors. Despite the different amplification levels of EGFR, or p53 status, in the various cell lines and GBM cells used in this study, Dvl2 depletion induces a block of proliferation and a morphologic change that has the characteristics of a differentiation-like phenotype. The fact that this phenotype was not observed upon a block of Wnt/β-catenin pathway suggests that more than just the well-characterized canonical Wnt signaling is involved downstream to Dvl in this process.

Mutations in components of noncanonical Wnt signaling have not been reported so far in human gliomas, though expression of the noncanonical Wnt ligand, Wnt5a, has been reported to increase with glioma grade (24, 46). We report here that deletion of Wnt5a blocks proliferation and induces senescence of glioma cells, showing that a noncanonical Wnt signaling pathway is required to maintain the proliferative capacity of glioma cells. Therefore, we show that both canonical and noncanonical Wnt signaling pathways maintain the proliferative capacity of human glioblastoma cells. Because loss of Dvl2 not only inhibits proliferation but also promotes a differentiation-like program, we conclude that inhibition of both Wnt signaling pathways might be required to initiate differentiation, though it is possible that other signaling pathways downstream to Dvl2 are also involved. The identification of Wnt5a as an essential player in maintaining the proliferative capacity of glioma cells may provide new therapeutic opportunities for treating this disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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In Memoriam

This study is dedicated to Agata Annino, a dear colleague who died of brain cancer at the age of 32.

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